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(54) Vector containing a chicken b-actin gene promoter for the expression of a desired gene.

(55) An expression vector for the expression of a desired gene in an animal cell is described, which contains a chicken β -actin gene promoter or a derivative thereof and downstream thereof a restriction enzyme site for the incorporation of a desired gene. Furthermore corresponding recombinant expression vectors, transformed microorganisms and processes for the expression of a desired gene in a host cell are described.

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VECTOR CONTAINING A CHICKEN β -ACTIN GENE PROMOTER FOR THE EXPRESSION OF A DESIRED GENE

The present invention relates to an expression vector for the expression of desired genes in animal cells, more particularly, it relates to an expression vector which contains a chicken β -actin gene promoter or a modified promoter thereof and is useful for expressing any desired gene efficiently in animal cells.

In recent years, with the progress of the gene engineering techniques, the techniques for producing a useful material in host cells has rapidly been advanced. For expression of a desired gene utilizing the genetic engineering techniques, a suitable host cell and an expression vector compatible with it are employed. As the host cell for expression, there have hitherto been widely studied microorganisms which can be easily handled such as *E. coli* and yeast. However, in recent years, it has been confirmed that the use of these microorganisms is limited in a certain degree in expression of a part of desired genes, and thus, an expression system which employs cultured cells of higher animals as the host cell has extensively been studied.

Such an expression system employing animal cells as the host cell has already been reported, including an expression system using a variety of animal viral gene promoters or animal cell gene promoters. The animal viral gene promoters include, for example, an SV 40 gene promoter, an adenovirus major late gene promoter or a hepatitis B virus gene promoter. The animal cell gene promoters include, for example, a thymidinekinase (tk) gene promoter, a metallothionein gene promoter, an interferon gene promoter or an immunoglobulin gene promoter.

Among the above promoters, it has been found that especially the SV 40 early gene promoter, the SV 40 late gene promoter and the adenovirus major late gene promoter have a powerful promoter activity. However, they are still insufficient for an industrial scale production and therefore more powerful, and efforts have been made to find highly expressionalable promoters.

Thus, the technical problem underlying the present invention is to provide an expression vector which is powerful and capable of expressing any desired gene in an industrial scale. This technical problem is solved by providing an expression vector containing a chicken β -actin gene promoter or a derivative thereof. When any desired gene is incorporated into this vector, it can be expressed at an extremely higher level than with conventional promoters. The desired gene can be any gene of interest which is either a gene already occurring in the host cell that is to be expressed in a higher level or which is exogenous with respect to the host cell, i.e. does not naturally occur therein. The latter alternative is preferred.

In a preferred embodiment of the above expression vector the derivative of the chicken β -actin promoter is a DNA fragment of the chicken β -actin promoter containing at its 3'-end at least the nucleotides up to the C at position -5 calculated from the ATG triplet of the original chicken β -actin gene. In a further preferred embodiment the derivative of the chicken β -actin promoter is a novel hybrid promoter which has a still more powerful promoter activity. It has been found that this novel hybrid promoter can express a desired gene at an expression level several to about ten times higher than the natural chicken β -actin gene promoter.

Thus, the present invention provides an expression vector containing a chicken β -actin gene promoter or a derivative thereof which can express a desired gene at an extremely high level in an expression system employing an animal cell.

Fig. 1 shows a DNA sequence of a chicken β -actin gene promoter;
 Fig. 2 shows a DNA sequence recognized by the restriction enzyme NcoI;
 Fig. 3 shows a DNA sequence after cleavage with the restriction enzyme NcoI;

Fig. 4 shows the position of a HindIII linker incorporated downstream of a promoter in plasmid p28 constructed in Example 1;

Fig. 5 shows a DNA sequence of a hybrid promoter comprising a chicken β -actin gene promoter and a rabbit β -globin gene, the hybrid promoter being contained in plasmid pAG-2 as constructed in Example 8;

- Fig. 6 shows the structure of plasmid pAc-2;
- Fig. 7 shows the structure of plasmid pSAC-2;
- Fig. 8 shows the structure of plasmid pAS-2;
- Fig. 9 shows the structure of plasmid pAR-2;
- Fig. 10 shows the structure of plasmid pKCR;
- Fig. 11 shows the structure of plasmid pAG-2;
- Fig. 12 shows the structure of plasmid pS-1;
- Fig. 13 shows the structure of plasmid pS-2;
- Fig. 14 shows the structure of plasmid pAcS-2;

Fig. 15 shows the structure of plasmid pARS-2; and
 Fig. 16 shows the structure of plasmid pAGS-2.

β -Actin is present in all cells and associated with a variety of cellular functions. It is one of major structural proteins ranging from protozoa to eukaryotes including human beings and the amino acid sequences thereof are extremely homologous to each other.

In relation to an expression system using a β -actin gene promoter other than the chicken β -actin gene promoter, there has been known a process for preparing a protein using a vector containing a human β -actin gene promoter [P. Gunning et al., Proc. Natl. Acad. Sci. USA, 84:4831-4835 (1987)]. However, according to this report, the promoter activity is about 1.7 times higher than that of the SV 40 early gene promoter and this is still insufficient from a practical point of view. In recent years, the promoter activity of the human β -actin gene promoter has been compared in various host cells [Gene 65, 135-139 (1988)]. This report shows that the human β -actin gene promoter demonstrated, in mouse-derived cells, a rather powerful activity in comparison to the SV 40 early promoter but, in human-derived cells or monkey-derived cells, a lower activity than the SV 40 early promoter.

On the contrary, the chicken β -actin gene promoter of the present invention exhibits a promoter activity that is at least 5 to 10 times higher than that of the well known SV 40 early promoter and shows a powerful activity not only in mouse-derived cells (e.g. L cells) but also in hamster-derived cells (e.g. CHO cells), African green monkey-derived cells (e.g. COS cells) and other animal cells.

The chicken β -actin gene promoter used in the present invention is a gene fragment containing the DNA sequence of Fig. 1. A chicken β -actin gene has already been cloned by T. A. Kost et al. [Nucleic Acids Research 11, No. 23, 8286-8287 (1983)].

The chicken β -actin gene promoter used in the expression vector of the present invention for the expression of a desired gene is a gene fragment having a high content of G (guanine) and C (cytosine) as a whole and containing sequences characteristic for a promoter such as the TATA box [Ann. Res. Biochem., 50, 349-383 (1981)] and the CCAAT box [Nucleic Acids Research 8, 127-142 (1980)].

In the DNA sequence of the promoter shown in Fig. 1, the DNA region from -909 to -7 G is considered to be a region (intron) to be deleted (spliced) after transcription into messenger RNA.

According to a preferred embodiment of the present invention a DNA fragment of the chicken β -actin promoter containing at its 3'-end at least the nucleotides up to the C at position -5 calculated from the ATG tripplet of the original chicken β -actin gene is incorporated into a vector and used as the chicken β -actin gene promoter. It is generally known that the promoter activity is little affected by deleting a part of the DNA sequence (e.g. up to about -30 bp) upstream of the initiation codon (ATG) of the original structural gene of β -actin. In case of the chicken β -actin gene promoter, however, it was confirmed by the present inventors that the desired gene can be expressed at a higher level by using a promoter which contains the base pairs up to the cytosine (C), that is located five base pairs upstream of the initiation codon (ATG) of the original structural β -actin gene.

The modified β -actin gene promoter used in the present invention also includes a hybrid promoter formed by incorporating a second promoter into an intron region of the above chicken β -actin gene promoter. The hybrid promoter of the present invention is a novel promoter which can express a desired gene at an extremely high level. It shows synergistic effect. The incorporated second promoter acts as an enhancer for the β -actin gene promoter while the β -actin gene promoter also acts as an enhancer for the incorporated second promoter. In the present specification, the "second promoter" is a promoter other than the chicken β -actin gene promoter, which functions in animal cells. Preferably, the "second promoter" is a promoter derived from a virus infectious for animal cells, and more preferably, it is the SV 40 early promoter or an LTR of the Rous sarcoma virus that is used for the construction of the above hybrid promoter. Such hybrid promoters of the present invention show a surprisingly powerful promoter activity, which is still several to 10 times higher than that of the chicken β -actin gene promoter which itself already has a powerful promoter activity.

The hybrid promoter as mentioned above can be prepared by incorporating the second promoter into the intron region of the chicken β -actin gene promoter. In this case, the incorporation of the second promoter is carried out in such a manner that the initiation codon (ATG) is not present in the DNA sequence ranging from downstream (3') of the site into which the second promoter is incorporated to the splicing acceptor region. Most preferably, the second promoter is incorporated into the MboII site of the chicken β -actin gene promoter.

when the second promoter is incorporated into the intron region of the chicken β -actin gene promoter as mentioned above, the hybrid chicken β -actin gene promoter preferably contains at its 3'-end the DNA sequence up to at least cytosine (C) at position -5 of Fig. 1.

The modified chicken β -actin gene promoter of the present invention further includes a hybrid promoter

which is constructed by deleting all DNA sequences downstream of a position in the intron region of the chicken β -actin gene promoter including the splicing acceptor sequence, and replacing it by a DNA sequence containing another splicing acceptor sequence. Such a desired DNA sequence or gene containing a splicing acceptor sequence includes, for example, a gene fragment of the rabbit β -globin gene containing a splicing acceptor sequence. In this way, by replacing the splicing acceptor sequence of the chicken β -actin gene promoter with another splicing acceptor sequence, the promoter activity of the chicken β -actin gene promoter can be further enhanced.

For linking a gene containing another splicing acceptor sequence to the promoter of the chicken β -actin gene, a DNA sequence downstream of a position in the intron region of the chicken β -actin gene promoter is deleted and replaced by a DNA sequence containing another splicing acceptor sequence. Such a deletion and replacement is preferably carried out at the Mbo II site as mentioned above.

Preferably the expression vector of the present invention further contains a suitable restriction enzyme site downstream of chicken β -actin gene promoter or the derivative thereof into which a desired gene can be incorporated. Such a restriction enzyme site may contain a recognition site of a single restriction enzyme or recognition sites of two or more restriction enzymes for facilitating an incorporation of various desired genes. In order to express the desired gene more efficiently, the expression vector of the present invention may further contain a polyadenylation sequence which is incorporated downstream of the site for the incorporation of the structural gene to be expressed. For cloning a transformant cell, a suitable marker gene may also be incorporated into the vector.

The expression vector of the present invention may further contain a gene derived from E. coli for cloning in E. coli. Such gene derived from E. coli includes, for example, a replication origin for E. coli, a suitable drug resistance gene which acts as a selection marker in cloning (e.g. an ampicillin or tetracycline resistance gene). When a gene derived from the plasmid pBR322 is incorporated into the vector, it is preferred to delete a toxic sequence inhibiting a replication of the vector in a host cell which resides at around the replication origin (ori) of pBR322 [Nature 293, 79-81 (1981)].

when a cell producing the large T antigen of SV 40, for example, a COS cell (derived from African green monkey kidney) is employed as the host cell for the expression of the desired gene, a replication origin which functions in this animal cell (e.g. SV 40 ori) may be further incorporated into the expression vector of the present invention. This increases the efficiency of the expression of the desired gene. Examples of the preferred expression vector of the present invention employing the chicken β -actin gene promoter are pAc-2 (Fig. 6) which can be used when a mouse L cell is used as the host cell and pSAC-2 (Fig. 7) which is effectively used when a COS cell is used as the host cell.

In order to enhance the expression efficiency of a desired gene, a dihydrofolate reductase (DHFR) gene may also be incorporated into the expression vector of the present invention, or alternatively, a co-transfection may be conducted with a DHFR expression plasmid. In this case, a DHFR gene-defective cell is preferably employed as the host cell and an addition of methotrexate to the culture medium amplifies the gene in a transformed cell, which provides a higher expression of the gene. Although such a method for enhancing the expression efficiency using a DHFR gene is already known, this can be applied to the expression vector of the present invention so that the expression efficiency is greatly improved.

Since β -actin is present in a variety of animal cells, the expression system of the present invention is applicable to a wide range of host cells with an extremely high expression efficiency, which is quite useful from an industrial point of view.

The expression vector of the present invention can provide an extremely high expression level which hitherto has never been achieved, and is useful for the expression of a desired gene even in an industrial-scale production.

The present invention is more specifically illustrated by the following Examples wherein the β -galactosidase gene of E. coli and a hepatitis B surface antigen (HBs) gene are employed as the desired gene, but should not be construed to be limited thereto.

Phages, plasmids, DNA, various enzymes, E. coli, cultured cells and the like were treated in the Examples by the procedures described in the following text books and magazines:

1. MOLECULAR CLONING A LABORATORY MANUAL ed. by T. MANIATIS et al. (1982), COLD SPRING HARBOR LABORATORY
2. METHODS IN ENZYMOLOGY 65, ed. by L. GROSSMAN et al. (1980), ACADEMIC PRESS
3. DNA cloning ed. by D. M. Glover et al. (1985) IRL PRESS.

55 The following abbreviations are employed in the Examples.

CAT: Chloramphenicol acetyl transferase

IacZ: β -Galactosidase (β -gal) structural gene

Example 1 (Construction of expression vectors, pAc-2 and pSAC-2)(1) Preparation of a promoter region of a chicken β -actin gene promoter:

5 A plasmid pAZ1037 which contains a first exon, a first intron and a part of a second exon of a chicken β -actin gene promoter and a CAT gene linked thereto [Nature 314, 286-289 (1985)] was digested with restriction enzyme Ncol (NEB #193). The restriction enzyme Ncol recognizes a sequence of six base pairs as shown in Fig. 2 and cleaves the sequence to form a sequence with 5' overhanging as shown in Fig. 3. In
 10 the present invention, the following procedures were carried out so that the splicing region between the first intron and the second exon of the chicken β -actin gene promoter correctly functions when used as the expression vector.

That is, the Ncol-digested DNA was treated with S1 nuclease (Takara #2410A) to delete the 5' overhanging region and only one base pair adjacent thereto. In this reaction, a sample DNA (10 μ g) was
 15 treated with 150 units of S1 nuclease in a solution (80 μ l) of 30 mM sodium acetate, pH 4.6, 100 mM NaCl, 1 mM ZnSO₄ at 37°C for 1 to 4 minutes.

After treating the DNA with S1 nuclease, the treated DNA was further treated with T4 DNA polymerase (Takara #2040A) to modify the single strand moiety of the DNA and thereto was linked a synthesized DNA pCCAAGCTTGG 5' end of which is phosphorylated (pHindIII linker, NEB #1050) with T4 DNA ligase (Takara #2011A) to cyclize. E. coli HB101 strain was transformed with the obtained DNA solution. After separating a single colony, a plasmid DNA in the transformed cells was collected and digested with restriction enzymes HindIII (NEB #104) and NarI (NEB #191). 6% of acrylamide gel electrophoresis was conducted and a clone
 20 was selected which contained a DNA fragment with a suitable size. The DNA fragment was cloned into the SalI-KpnI site of phage vector M13mp19 (NEB #400-19) and a DNA sequence about the HindIII site was determined by a dideoxy method [Proc. N.A.S. 74, 5463-5477 (1977)] to screen the desired clone.

The thus obtained plasmid clone p28, as shown in Fig. 4, retained a DNA sequence ranging from the splicing region to the structural gene of the natural chicken β -actin gene and had a structure where a gene at 3' end from the initiation codon (ATG) was deleted and thereto HindIII site was incorporated. Following the same procedure, clone p29, where up to 2 base pairs upstream the ATG were deleted, and clone p3,
 30 where up to 20 base pairs upstream the ATG were deleted, were also obtained.

(2) Construction of expression vector, pAc-2:

35 A plasmid pSV2-cat containing a splicing region for SV 40 early transcription and a polyadenylation signal [Molecular Cell Biology 2, 1044,1051 (1982)] was digested with restriction enzyme MfII (Takara #1070). The cleaved sites were modified with T4 DNA polymerase and thereto was linked a phosphorylated HindIII linker with T4 DNA ligase. The obtained plasmid was further digested with restriction enzymes HindIII and BamHI (NEB #136) and the cleaved fragments were subjected to 6% of acrylamide gel
 40 electrophoresis to extract a HindIII-BamHI fragment of about 900 bp. The plasmid p28 obtained by the above procedure (1) was digested with restriction enzymes HindIII and BamHI and dephosphorylated with alkaline phosphatase derived from calf intestine (Takara #2250A). This was linked to the above HindIII-BamHI fragment of about 900 bp with T4 DNA ligase to cyclize to give a plasmid pAc-2 (Fig. 6).

45 (3) Construction of an expression vector, pSAC-2:

The same plasmid pSV2-cat as used in the above procedure (2) was digested with restriction enzymes AccI (NEB #161) and SphI (NEB #182) and the cleaved sites were blunt-ended with T4 DNA polymerase.
 50 This was linked and cyclized in the presence of a phosphorylated XbaI linker (NEB #1032) with T4 DNA ligase to prepare a plasmid pSV-cat-delE [Proc. Natl. Acad. Sci. USA 83, 9537-9541 (1986)].

This plasmid pSV-cat-delE was digested with restriction enzyme HindIII and the cleaved sites were modified with T4 DNA polymerase. This was linked and cyclized in the presence of a phosphorylated XbaI linker (NEB #1030) with T4 DNA ligase. The obtained plasmid was further digested with restriction enzymes EcoRI (NEB #101) and XbaI (NEB #146) and the cleaved fragments were subjected to 1% of agarose gel electrophoresis to extract an EcoRI-XbaI fragment of about 2 kbp. The plasmid pAc-2 formed in the above procedure (2) was digested with restriction enzymes XbaI and EcoRI to give an XbaI-EcoRI fragment of about 2.2 kbp, to which was linked the above EcoRI-XbaI fragment of about 2 kbp to cyclize to prepare a

plasmid pSAC-2 (Fig. 7).

Example 2 (Construction of β -galactosidase expression plasmids pAc-lacZ and pSAC-lacZ)

5

(1) Preparation of a β -galactosidase gene fragment:

A plasmid pCH110 [Pharmacia #27-4508-01], which contained whole lacZ gene encoding β -galactosidase, was digested with restriction enzymes HindIII and BamHI and the cleaved fragments were subjected to 1% of agarose gel electrophoresis to prepare a HindIII-BamHI fragment of about 3.8 kbp. This fragment contained a splicing region and a polyadenylation region of SV 40 early gene transcript.

15 (2) Construction of plasmid pAc-2:

The plasmid p28 constructed in Example 1 (1) was digested with restriction enzymes HindIII and BamHI and the cleaved sites were dephosphorylated with alkaline phosphatase derived from calf intestine. To this was linked the HindIII-BamHI fragment of about 3.8 kbp obtained in the above procedure (1) to construct a plasmid pAc-lacZ.

20 Following the same procedure, plasmids pAc-lacZ(29) and pAc-lacZ(3) were constructed from the plasmids p29 and p3 obtained in Example 1 (1), respectively.

25 (3) Construction of pSAC-lacZ:

The plasmid pSAC-2 constructed in Example 1 (3) was digested with restriction enzymes HindIII and BamHI and the cleaved sites were dephosphorylated with alkaline phosphatase derived from calf intestine. To this was linked the HindIII-BamHI fragment of about 3.8 kbp obtained in the above procedure (1) with T4 DNA ligase to cyclize to construct a plasmid pSAC-lacZ.

Example 3 (Construction of Hepatitis B surface antigen (HBsAg) expression vector pSAC-HBs)

35 A plasmid pAS101 containing a repressible acid phosphatase promoter and being capable of producing HBsAg after transformation of yeast (Japanese Patent Second Publication No. 55951/1984) was digested with restriction enzyme Xhol and the cleaved fragments were subjected to 6 % of acrylamide gel electrophoresis to extract a DNA fragment containing HBsAg gene of about 1.3 kbp.

40 On the other hand, the plasmid pSAC-2 constructed in Example 1 (3) was digested with restriction enzyme SalI (Takara #1080A) and the cleaved sites were dephosphorylated with alkaline phosphatase derived from calf intestine. This was linked to the above DNA fragment of about 1.3 kbp containing HBsAg gene with T4 DNA ligase to cyclize and prepared an HBsAg expression plasmid pSAC-HBs.

Next, a control expression plasmid pSVE-HBs was constructed for evaluation of an expression of HBsAg in the following procedure.

45 The above plasmid pAS101 (10 μ g) was reacted with restriction enzyme Xhol (10 U) at 37 °C for 4 hours and the obtained fragments were subjected to 0.75 % of agarose gel electrophoresis. 1.3 Kb band containing an HBsAg gene was separated from the agarose gel and put in a dialysis tube, which was again subjected to an electrophoresis. After elution of DNA from the gel fragment, only a DNA solution was taken out from the dialysis tube and DNA was extracted by an ethanol precipitation. The extracted DNA fragment 50 containing HBsAg gene (1 μ g) was reacted with T4 DNA polymerase (1 U) at 37 °C for 30 minutes. Treatment with phenol and ethanol precipitation were conducted to extract DNA.

On the other hand, a plasmid pKSV-10 containing an SV 40 early gene promoter (Pharmacia #27-4926-01) (1 μ g) was reacted with restriction enzyme BglII (1 U) at 37 °C for 1 hour. DNA was extracted by phenol treatment and ethanol precipitation. The extracted DNA was treated with T4 DNA polymerase in the same manner as mentioned above. A mixture of the HBsAg gene fragment which was blunt-ended with T4 DNA polymerase reaction (500 ng) and pKSV-10 (50- ng) was reacted in the presence of T4 DNA ligase (1 U) at 4 °C for 12 hours. *E. coli* HB101 was transformed with this reaction mixture. A plasmid was extracted from the transformant to give a plasmid pSVE-HBs where the HBsAg gene was incorporated into pKSV-10.

Example 4 (Production of β -galactosidase in COS cell and L-cell)

COS cells of L-cells were plated on 100 mm round Petri dish at 1×10^5 cells/dish and each 20 μ g of the following plasmids DNA was introduced into these host cells by a calcium phosphate method in accordance with the conventional procedure.

The cells were contacted with calcium phosphate-gel for 24 hours and further cultured in a 10% FCS-DME for 24 hours. The cells were peeled off the dish with trypsin treatment and pelleted by a low-speed centrifugation. The pellet was suspended in F-T buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA) 200 μ l) and a freezing-thawing procedure was repeated for three times, followed by centrifugation to collect a supernatant.

10 μ l of the thus obtained cell extract (200 μ l) were used and a β -gal activity was measured. The measurement of β -gal activity was conducted by detecting a change in absorbance at 420 nm accompanied with a color development of ONPG (o-nitrophenyl- β -D-galactopyranoside) in the conventional manner. Table 1 shows a relative value where the absorbance shown by the cell extract of COS-cells or L-cells transformed with the plasmid pCH110 containing an SV 40 early promoter [Pharmacia #27-4508-01] was made 1.

Table 1

	Plasmid	β -Gal Activity	
		COS-cell	L-cell
(1)	pCH110	1.0	1.0
(2)	pAc-1acZ	4.0	5.0
(3)	pAc-1acZ(3)	1.2	-
(4)	pAc-1acZ(29)	2.7	-
(5)	pSAC-1acZ	57.2	3.0
(6)	pSAC-2	0.01	0.01

30

As shown in Table 1, the plasmids pAc-lacZ and pSAC-lacZ, which contained the chicken β -actin gene promoter, showed 3 to 5 times higher values in L-cell than the plasmid pCH110 which contained the SV 40 early gene promoter. Since the SV 40 ori functioned effectively, the plasmid pSAC-lacZ showed more than 35 50 times higher values in COS-cell than the plasmid pCH110.

Example 5 (Production of HBsAg in COS-cell)

40 COS-cells (1×10^5 cells/well) were plated on a Falcon 6-well plate for cell culture and, in accordance with the conventional DEAE-Dextran method, the following plasmid DNAs (each 5 μ g) were introduced into the cells. Three days after introduction of DNA, the culture supernatant three days after introduction of DNA was collected with CellPfect Transfection Kit (Pharmacia) and an HBsAg activity was measured with HBsAg detection kit "Auslia II" (manufactured by Dainabott). The results are shown in Table 2.

45 As shown in Table 2, the plasmid pSAC-HBs, which contained the chicken β -actin gene promoter, showed 3 to 5 times higher values than the plasmid pSVE-HBs which contained the SV 40 early gene promoter.

50

55

Table 2

Plasmid		HBsAg Activity (cpm)
(1)	pSVE-HBs	1,100
(2)	pSAc-HBs	6,800
(3)	pSAc-2	300

15 Example 6 (Preparation of expression vector containing hybrid promoter of the chicken β -actin gene promoter and the SV 40 early promoter; pSA-lacZ and pAS-2)

20 The plasmid pSV2-cat used in Example 1 (2) was digested with restriction enzymes PvuII (Takara #1076A) and HindIII and the cleaved fragments were subjected to 6 % of acrylamide gel electrophoresis to prepare a PvuII-HindIII fragment of about 340 bp containing the SV 40 early promoter. The PvuII-HindIII fragment was modified with T4 DNA polymerase at its termini and thereto was linked a phosphorylated XbaI linker with T4 DNA ligase. After digesting the ligate with restriction enzyme XbaI (Takara #1093A), the cleaved fragments were subjected to 6 % of acrylamide gel electrophoresis to prepare a XbaI-XbaI fragment of about 350 bp.

25 The plasmid pSAc-2 constructed in Example 1 (3) was digested with restriction enzymes XbaI and PstI and the cleaved fragments were subjected to 1 % of agarose gel electrophoresis to prepare a XbaI-PstI fragment of about 1.6 kbp. This fragment was digested with restriction enzyme MboI (NEB #148) and the cleaved sites were modified with T4 DNA polymerase and thereto was linked a phosphorylated XbaI linker with T4 DNA ligase. The ligate was digested with restriction enzymes XbaI and HindIII and the cleaved fragments were subjected to 1 % of agarose gel electrophoresis to prepare a XbaI-HindIII fragment of about 1.3 kbp.

30 The plasmid pAc-lacZ constructed in Example 2 was digested with restriction enzymes XbaI and HincII and the cleaved fragments were subjected to 1 % of agarose gel electrophoresis to prepare a XbaI-HincII fragment of about 5.8 kbp. This fragment was mixed with the above XbaI-HindIII fragment of about 1.3 kbp and, after digestion with restriction enzyme XbaI, ligated to each other with T4 DNA ligase to cyclize and construct a plasmid pAc-lacZ-XbaI.

35 This plasmid pAc-lacZ-XbaI was digested with restriction enzyme XbaI and thereto was linked the above XbaI-XbaI fragment of about 350 bp with T4 DNA ligase to construct a plasmid pAS-lacZ.

40 The plasmid pAc-2 constructed in Example 1 (2) was digested with restriction enzymes HindIII and BamHI and the cleaved fragments were subjected to 1 % of agarose gel electrophoresis to extract a HindIII-BamHI fragment of about 900 bp, which contained the splicing region and the poly adenylation signal of the SV 40 early promoter.

45 The plasmid pAS-lacZ was digested with restriction enzymes HindIII and BamHI and the cleaved fragments were subjected to 1 % of agarose gel electrophoresis to prepare a HindIII-BamHI fragment of about 3.7 kbp. To this HindIII-BamHI fragment of about 3.7 kbp was linked the above HindIII-BamHI fragment of about 900 bp with T4 DNA ligase to construct a plasmid pAS-2 (Fig. 2).

50 Example 7 (Preparation of expression vector containing hybrid promoter of the chicken β -actin gene promoter and the RSV-LTR promoter; pAR-lacZ and pAR-2)

55 A plasmid pRSV-cat containing LTR of RSV [Proc.Natl. Acad. Sci. USA. 79, 6777-6781 (1982)] was digested with restriction enzymes NruI (NEB #192) and TaqI (NEB #149) and the cleaved fragments were subjected to 6 % of acrylamide gel electrophoresis to prepare a NruI-TaqI fragment of about 340 bp. This NruI-TaqI fragment was modified with T4 DNA polymerase at its termini and thereto was linked a phosphorylated XbaI linker. After digesting the ligate with restriction enzyme XbaI, the cleaved fragment was subjected to 6 % of acrylamide gel electrophoresis to prepare a XbaI-XbaI fragment of about 350 bp.

The plasmid pAc-lacZ-XbaI constructed in Example 6 was digested with restriction enzyme XbaI and

thereto was linked the above XbaI-XbaI fragment of about 350 bp with T4 DNA ligase to construct a plasmid pAR-lacZ.

The plasmid pAR-lacZ was digested with restriction enzymes HindIII and BamHI and the cleaved fragments were subject to 1 % of agarose gel electrophoresis to prepare a HindIII-BamHI fragment of about 3.7 kbp. To this HindIII-BamHI fragment of about 3.7 kbp was linked the HindIII-BamHI fragment of about 900 bp prepared in Example 6 with T4 DNA ligase to construct a plasmid pAR-2 (Fig. 9).

Example 8 (Preparation expression vector containing hybrid promoter of the chicken β -actin gene promoter and the rabbit β -globin gene; pAG-lacZ and pAG-2)

A plasmid pKCR containing a rabbit β -globin gene from the middle of the second exon to the middle of the third exon thereof [Proc. Natl. Acad. Sci. USA, 78, 1527-1531 (1981)] (Fig. 10) was digested with restriction enzyme Apal (NEB #Apal) and the cleaved sites were modified with T4 DNA polymerase and thereto was linked a phosphorylated XbaI linker with T4 DNA ligase. This ligate was digested with restriction enzyme EcoRI and the cleaved sites were modified with DNA polymerase I Large Fragment (NEB #210). Thereto was linked a pHindII linker (NEB #1022) with T4 DNA ligase and the ligate was digested with restriction enzymes XbaI and HindIII, followed by 6 % of acrylamide gel electrophoresis to prepare a XbaI-HindIII fragment of about 90 bp which contained a splicing acceptor site in the second intron of the rabbit β -globin gene.

The plasmid pAc-lacZ-XbaI constructed in Example 6 was digested with restriction enzymes XbaI and HindIII and the cleaved fragments were subjected to 1 % of agarose gel electrophoresis to prepare a XbaI-HindIII fragment of about 7 kbp. Thereto was linked the above XbaI-HindIII fragment of about 90 bp with T4 DNA ligase to construct a plasmid pAG-lacZ.

This plasmid pAG-lacZ was digested with restriction enzymes HindIII and BamHI and the cleaved fragments were subjected to 1 % of agarose gel electrophoresis to prepare a HindIII-BamHI fragment of about 3.3 kbp. To this HindIII-BamHI fragment of about 3.3 kbp was linked the above-prepared HindIII-BamHI fragment of about 900 bp with T4 DNA ligase to construct a plasmid pAG-2 (Fig. 11).

Example 9 (Construction of expression vector containing SV 40 ori; pAcS-lacZ, pAcS-2, pARS-lacZ, pARS-2, pAGS-lacZ and pAGS-2)

The plasmid pSV2-cat used in Example 1 (2) was digested with restriction enzymes HpaI (NEB #105) and HindIII and the cleaved sites were modified with T4 DNA polymerase and ligated to each other with T4 DNA ligase to cyclize and prepare a plasmid pS-1 (Fig. 12).

This plasmid pS-1 was digested with restriction enzyme SphI and the cleaved sites were modified with T4 DNA polymerase and thereto was linked a phosphorylated BamHI linker (NEB #1021) with T4 DNA ligase to cyclize and prepare a plasmid pS-2 (Fig. 13).

This plasmid pS-2 was digested with restriction enzyme BamHI and the cleaved fragments were subjected to 6 % of acrylamide gel electrophoresis to give a BamHI-BamHI fragment of about 350 bp which contained a polyadenylation signal of SV 40 early transcription.

The plasmid pAc-lacZ constructed in Example 2 was digested with restriction enzyme BamHI and thereto was linked the above BamHI-BamHI fragment of about 350 bp with T4 DNA ligase to cyclize and construct a plasmid pAcS-lacZ.

The plasmid pAc-2 constructed in Example 1 (2) was digested with restriction enzyme BamHI and thereto was linked the above BamHI-BamHI fragment of about 350 bp with T4 DNA ligase to cyclize and construct a plasmid pAcS-2 (Fig. 14).

The plasmid pAR-lacZ constructed in Example 7 was digested with restriction enzyme BamHI and thereto was linked the above BamHI-BamHI fragment of about 350 bp with T4 DNA ligase to cyclize and construct a plasmid pARS-lacZ.

In the same manner as mentioned above, there were constructed a plasmid pARS-2 (Fig. 15) from the plasmid pAR-2 constructed in Example 7, a plasmid pAGS-lacZ from the plasmid pAG-lacZ constructed in Example 8, and a plasmid pAGS-2 (Fig. 16) from the plasmid pAG-2 constructed in Example 8, respectively.

Example 10 (Production of β -galactosidase with expression vector containing modified chicken β -actin gene

promoter)

COS cells or L-cells were plated on 100 mm round Petri dish at 1×10^6 cells/dish and each 20 µg of the various plasmids (pCH110, pAS-lacZ, pAR-lacZ, pAG-lacZ, pAcS-lacZ, pARS-lacZ, pAGS-lacZ) DNA was introduced into these host cells by a calcium phosphate method in accordance with the conventional procedure.

The cells were contacted with calcium phosphate-gel for 24 hours and further cultured in a 10 % FCS-DME for 24 hours. The cells were peeled off the dish with trypsin treatment and pelleted by a low-speed centrifugation. The pellet was suspended in F-T buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.5 10 mM EDTA) (200 µl) and a freezing-thawing procedure was repeated for three times, followed by centrifugation to collect a supernatant.

Using 10 µl of the thus obtained cell extract (200 µl) a β -gal activity was measured. The measurement of β -gal activity was conducted by detecting a change in absorbance at 420 nm accompanied with a color development of ONPG (o-nitrophenyl- β -D-galactopyranoside) in the conventional manner. Table 3 shows a relative value where the absorbance shown by the cell extract of COS-cells or L-cells transformed with the plasmid pCH110 containing the SV 40 early promoter [Pharmacia #27-4508-01] was made 1.

Table 3

	Plasmid	β -Gal Activity	
		COS-cell	L-cell
(1)	pCH110	1.0	1.0
(2)	pAS-1acZ	20.9	19.4
(3)	pAR-1acZ	11.5	17.0
(4)	pAG-1acZ	12.0	10.2
(5)	pAcS-1acZ	15.0	4.5
(6)	pARS-1acZ	55.8	23.9
(7)	pAGS-1acZ	55.8	7.4
	pAc-1acZ-XbaI	4.0	5.0
	pAc-1acZ	4.0	5.0

As is clearly shown in Table 3, the plasmid where the lacZ gene was incorporated into the expression vector containing the hybrid promoter of the present invention showed an extremely high expression as compared with the expression plasmid (pAc-lacZ) containing the natural chicken β -actin gene promoter.

Example 11 (Production of HBsAg with expression vector containing the modified chicken β -actin gene promoter)

(1) Construction of HBsAg-expression plasmids pAS-HBs, pAG-HBs, pAcS-HBs, pARS-HBs and pAGS-HBs:

The plasmid pAS101 (10 µg) used in Example 3 was reacted with restriction enzyme Xhol (10 U) at 37 °C for 4 hours and the cleaved fragments were subjected of 6 % of agarose gel electrophoresis. 1.3 Kb band containing HBs gene was cut off the agarose gel and put in a dialysis tube and further subjected to an electrophoresis. After DNA was eluted from the gel fragment, only a DNA solution was taken from the dialysis tube and DNA was extracted by an ethanol precipitation. The extracted DNA fragments (1 µg) containing HBsAg gene were reacted with T4 DNA polymerase (1 U) at 37 °C for 30 minutes. Then, phenol treatment and ethanol precipitation were conducted to extract DNA.

Next, six kinds of the plasmids constructed in Examples 6 to 9 (pAS-2, pAR-2, pAG-2, pAcS-2, pARS-2 and pAGS-2) were digested with restriction enzyme SalI and the cleaved fragments were dephosphorylated with alkaline phosphatase derived from calf intestine. Thereto was linked the above DNA fragment of about 1.3 kbp containing HBsAg gene with T4 DNA ligase to cyclize and construct an HBsAg-expression plasmids pAS-HBs, pAR-HBs, pAG-HBs, pAcS-HBs, pARS-HBs and pAGS-HBs, respectively.

As a reference expression plasmid for evaluation of HBsAg expression, the plasmid pSVES-HBs

constructed in Example 3 was used.

(2) Expression of HBs in COS-cells:

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COS-cells were plated on a Falcon 6-well plate for cell culture at 1×10^5 cells/well and, in accordance with the conventional procedure, each 5 µg of the following plasmid DNAs was introduced into the cells by an DEAE-Dextran method. Three days after introduction of DNA, a culture supernatant was separated with CellPfect Transfection Kit (Pharmacia) and an HBsAg activity was measured with HBsAg detection kit 10 "Ausilia II" (manufactured by Dainabott). The results are shown in Table 4.

Table 4

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	Plasmid	HBsAg Activity (cpm)
(1)	pAS-HBs	5100
(2)	pAR-HBs	3000
(3)	pAG-HBs	3100
(4)	pAcS-HBs	3500
(5)	pARS-HBs	6200
(6)	pAGS-HBs	7200
(7)	pSVE-HBs	1050

As shown in Table 4, the expression plasmids containing the modified chicken β -actin gene promoter showed an HBsAg activity higher than those of the plasmid pSVE-HBs containing the SV 40 early gene promoter.

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Claims

- 35 1. An expression vector for the expression of a desired gene in an animal cell, which contains a chicken β -actin gene promoter or a derivative thereof and downstream thereof a restriction enzyme site for the incorporation of a desired gene.
2. The expression vector of claim 1, wherein the derivative of the chicken β -actin promoter is a DNA fragment of the chicken β -actin promoter containing at its 3'-end at least the nucleotides up to the C at position -5 calculated from the ATG tripplet of the original chicken β -actin gene.
- 40 3. The expression vector of claim 1 wherein the derivative of the chicken β -actin gene promoter is a hybrid promoter formed by the incorporation of a second promoter into an Intron region of the chicken β -actin gene promoter.
4. The expression vector of claim 3 wherein said second promoter is a promoter derived from a virus which is infectious for animal cells.
- 45 5. The expression vector of claim 3 or 4 wherein said second promoter is a promoter derived from an SV 40 gene.
6. The expression vector of any one of claims 3 to 5 wherein said second promoter is an SV 40 early promoter.
- 50 7. The expression vector of claim 3 or 4 wherein said second promoter is an LTR of the Rous sarcoma virus.
8. The expression vector of any one of claims 3 to 7 wherein said promoter hybrid is constructed by replacing a DNA sequence downstream of a position in the Intron of the chicken β -actin promoter and including the splicing acceptor sequence by a DNA sequence containing another splicing acceptor sequence.
- 55 9. The expression vector of claim 8 wherein said other splicing acceptor sequence is a fragment of the rabbit β -globin gene containing a splicing acceptor sequence.
10. The expression vector of any of claims 1 to 9 which further contains a gene derived from an E. coli plasmid.

11. The expression vector of claim 10 wherein said gene derived from the E. coli plasmid is a gene fragment containing a drug resistance gene and a replication origin capable of functioning in E. coli.
12. The expression vector of any one of claims 1 to 11 which further contains an SV 40 early transcription splicing region and polyadenylation region.
- 6 13. The expression vector of any one of claims 1 to 12 wherein the SV 40 replication origin (SV 40 ori) is further incorporated.
14. The expression vector of any one of claims 1 to 13 which additionally contains a desired gene to be expressed in a host cell, preferably in an animal cell.
15. A microorganism transformed with an expression vector of any one of claims 1 to 14.
- 10 16. The microorganism of claims 15 which is an animal cell.
17. A process for expressing a desired gene in a host cell which comprises the incorporation of said gene into an expression vector according to any one of claims 1 to 14 at the restriction enzyme site provided therein for the incorporation of the desired gene, introducing said vector into an animal cell and culturing the obtained transformed animal cell under suitable conditions.

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Fig. 1-(1)

-1271 -1261 -1251 -1241
 TCGAG GTGAGCCCCA CGTTCTGCTT CACTCTCCCC

 -1231 -1221 -1211 -1201
 ATCTCCCCCC CCTCCCCCAC CCCAATTTG TATTTATTTA

 -1191 -1181 -1171 -1161
 TTTTTTAATT ATTTTGTCGA GCGATGGGGG CGGGGGGGGG

 -1151 -1141 -1131 -1121
 GGGGGCGCGC GCCAGGCAGG GCAGGGCGGG GCGAGGGCG

 -1111 -1101 -1091 -1081
 GGGCGGGGCG AGGCGGAGAG GTGCGGCCGG AGCCAATCAG

 -1071 -1061 -1051 -1041
 AGCGGGCGCGC TCCGAAAGTT TCCTTTATG GCGAGGCAGC

 -1031 -1021 -1011 -1001
 GGCGGGCGGC GCCCTATAAA AAGCGAAGCG CGCGGGCGGC

 -991 -981 -971 -961
 GGGAGTCGCT GCGTTGCCTT CGCCCCGTGC CCCGCTCCGC

 -951 -941 -931 -921
 GCCGCCTCGC GCCGCCCGCC CGGGCTCTGA CTGACCGCGT

 -911 -901 -891 -881
 TACTCCCACAC GGTGAGCGGG CGGGACGGCC CTTCTCCTCC

 -871 -861 -851 -841
 GGGCTGTAAT TAGCGCTTGG TTTAATGACCG GCTCGTTCT

Fig. 1-(2)

-831 -821 -811 -801
 TTTCTGTGGC TGC GTGAAAG CCTTAAAGGG CTCCGGGAGG

 -791 -781 -771 -761
 GCC CTTTGTC CGGGGGGGAG CGGCTCGGGG GGTGC GTGCG

 -751 -741 -731 -721
 TGTGTGTGTG CGTGGGGAGC GCC GCGT GCG GCC CGCGCTG

 -711 -701 -691 -681
 CCCGGCGGCT GTGAGCGCTG CGGGCGCGGC GC GGGGCTTT

 -671 -661 -651 -641
 GTGCGCTCCG CGTGTGCGCG AGGGGAGCGC GGCCGGGGGC

 -631 -621 -611 -601
 GGTGCCCGC GGTGCGGGGG GGCTGCGAGG GGAACAAAGG

 -591 -581 -571 -561
 CTGCGTGC GG GGTGTGTGCG TGGGGGGGTG AGCAGGGGGT

 -551 -541 -531 -521
 GTGGGCGCGG CGGT CGGGCT GTAACCCCCC CCTGCACCC

 -511 -501 -491 -481
 CCTCCCCGAG TTGCTGAGCA CGGCCCGGCT TC GGGTGC CGG

 -471 -461 -451 -441
 GGCTCCGTGC GGGCGTGGC CGGGGGCTCG CGTGC CGGG

 -431 -421 -411 -401
 CGGGGGGTGG CGGCAGGTGG GGGTGCCGGG CGGGGGCGGG

Fig. 1-(3)

-391 -381 -371 -361
 CCGCCTCGGG CGGGGGAGGG CTGGGGGAG GGGCGCGGC

-351 -341 -331 -321
 GCCCCGGAGC GCCGGCGGCT GTCGAGGCCGC GGCGAGGCCG

-311 -301 -291 -281
 AGCCATTGCC TTTTATGGTA ATCGTGCAG AGGGCGCAGG

-271 -261 -251 -241
 GACTTCCTT GTCCCAAATC TGGCGGAGCC GAAATCTGGG

-231 -221 -211 -201
 AGGCGCCGCC GCACCCCCCTC TAGCGGGCGC GGGCGAAGCG

-191 -181 -171 -161
 GTGCAGGAAGG GGCAGGAAGG AAATGGGCGG GGAGGGCCTT

-151 -141 -131 -121
 CGTGCAGTCGC CGCGCCGCCG TCCCCTCTC CATCTCCAGC

-111 -101 -91 -81
 CTCGGGGCTG CCGCAGGGGG ACGGCTGCCT TCAGGGGGGA

-71 -61 -51 -41
 CGGGGCAGGG CGGGGTTCGG CTTCTGGCGT GTGACCGGGCG

-31 -21 -11 -1
 GGGTTTATAT CTTCCCTTCT CTGTTCCCTCC GCAGCCAGCC (ATG)
 ↑ Mbo II recognition site
 Mbo II cleavage site

Fig. 2

CCATGG
GGTAC

Fig. 3

C
GGTAC

Fig. 4

β - actin promoter region β - actin structural gene

-51 -41 -31 -21 -1 -1
 CTTCTGGGT GTGACCGCG GGGTTATAT CTTCCTCTCTC CTGTTCTCC GCAGCCAGCC ATG GAT GAT ...
 HindIII linker

plasmid p 2 8 CTTCTGGGT GTGACCGCG GGGTTATAT CTTCCTCTCTC CTGTTCTCC GCAGCCAGCC ATG GAT GAT ...

Fig. 5-(1)

-1271 -1261 -1251 -1241
 TCGAG GTGAGCCCCA CGTTCTGCTT CACTCTCCCC

 -1231 -1221 -1211 -1201
 ATCTCCCCCC CCTCCCCACC CCCAATTTG TATTTATTTA

 -1191 -1181 -1171 -1161
 TTTTTTAATT ATTTTGTGCA GCGATGGGGG CGGGGGGGGG

 -1151 -1141 -1131 -1121
 GGGGGCGCGC GCCAGGCGGG GCGGGCGGG GCGAGGGCG

 -1111 -1101 -1091 -1081
 GGGCGGGGCG AGGCGGAGAG GTGCGGCAGC AGCCAATCAG

 -1071 -1061 -1051 -1041
 AGCGGCGCGC TCCGAAAGTT TCCTTTATG GCGAGGGCGC

 -1031 -1021 -1011 -1001
 GGCGGCCGGCG GCCCTATAAA AAGCGAAGCG CGCGGCCGGC

 -991 -981 -971 -961
 GGGAGTCGCT GCGTTGCCTT CGCCCCGTGC CCCGCTCCGC

 -951 -941 -931 -921
 GCCGCCTCGC GCCGCCCGCC CCGGCTCTGA CTGACCGCGT

 -911 -901 -891 -881
 TACTCCCAC A GGTGAGCGGG CGGGACGGCC CTTCTCCTCC

 -871 -861 -851 -841
 GGGCTGTAAAT TAGCGCTTGG TTTAATGACG GCTCGTTCT

 -831 -821 -811 -801
 TTTCTGTGGC TGC GTGAAAG CCTTAAAGGG CTCCGGGAGG

Fig. 5-(2)

-791 -781 -771 -761
 GCCCTTTGTG CGGGGGGGAG CGGCTCGGGG GGTGC GTGCG

 -751 -741 -731 -721
 TGTGTGTGTG CGTGGGGAGC GCCGC GTGCG GCCC GCGCTG

 -711 -701 -691 -681
 CCCGGCGGCT GTGAGCGCTG CGGGCGCGC GC GGGGGCTTT

 -671 -661 -651 -641
 GTGCGCTCCG CGTGTGCGC AGGGGAGCGC GGCCGGGGC

 -631 -621 -611 -601
 GGTGCCCGC GGTGCGGGGG GGCTGCGAGG GGAACAAAGG

 -591 -581 -571 -561
 CTGCGTGC GG GGTGTGTGCG TG GGGGGGTG AGCAGGGGT

 -551 -541 -531 -521
 GTGGGCGCGG CGGTGGCT GTAACCCCCC CCTGCACCCC

 -511 -501 -491 -481
 CCTCCCCGAG TTGCTGAGCA CGGCCCCGGCT TC GGGGTGCGG

 -471 -461 -451 -441
 GGCTCCGTG C GGGCGTGGC CGGGGGCTG CCGTGCCGGG

 -431 -421 -411 -401
 CGGGGGGTGG CGGCAGGTGG GGGTGCCGGG CGGGGGCGGG

 -391 -381 -371 -361
 CCGCCTCGGG CGGGGGAGGG CT CGGGGGAG GGGCGCGGCG

 -351 -341 -331 -321
 GCCCCGGAGC GCCGGCGGCT GTCGAGGGCGC GGCGAGCCG

Fig. 5-(3)

-311 -301 -291 -281
 AGCCATTGCC TTTTATGGTA ATCGTGCAGAG AGGGCGCAGG

-271 -261 -251 -241
 GACTTCCTTT GTCCCCAAATC TGGCGGAGCC GAAATCTGGG

-231 -221 -211 -201
 AGGCGCCCGCC GCACCCCCCTC TAGCGGGCGC GGGCGAAGCG

-191 -181 -171 -161
 GTGCGGCGCC GGCAGGAAGG AAATGGGCGG GGAGGGCCTT

-151 -141 -131 -121
 CGTGCCTCGC CGCGCCCGCC TCCCCTTCTC CATCTCCAGC

-111 -101 -91 -81
 CTCGGGGCTG CCGCAGGGGG ACGGCTGCCT TCAGGGGGGA

Chicken s-actin gene ←
 -71 -61 -51 -40
 CGGGGCAGGG CGGGGTTCTGG CTTCTGGCGT GTGACCGGGCGG

→ β-globin gene
-CTCTAGAG- CCTCTGCTAACCATGTTATGCCTTCTTCTT
 Xba I recognition site

TCCTACAGCTCTGGCAACGTGCTGGTTGTGCTGTCTCA
 ↑ splicing acceptor site

TCATTTGGCAAAGAATTCAAGCTT
 Hind III recognition site

Fig. 6

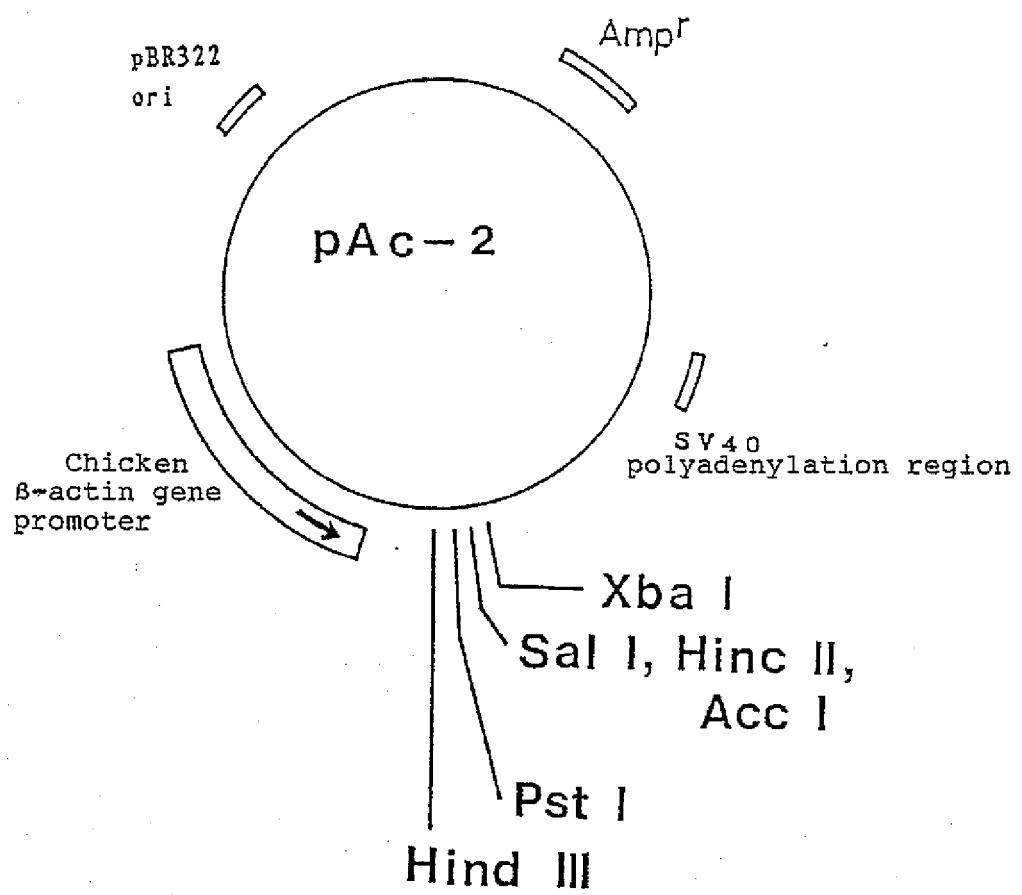


Fig. 7

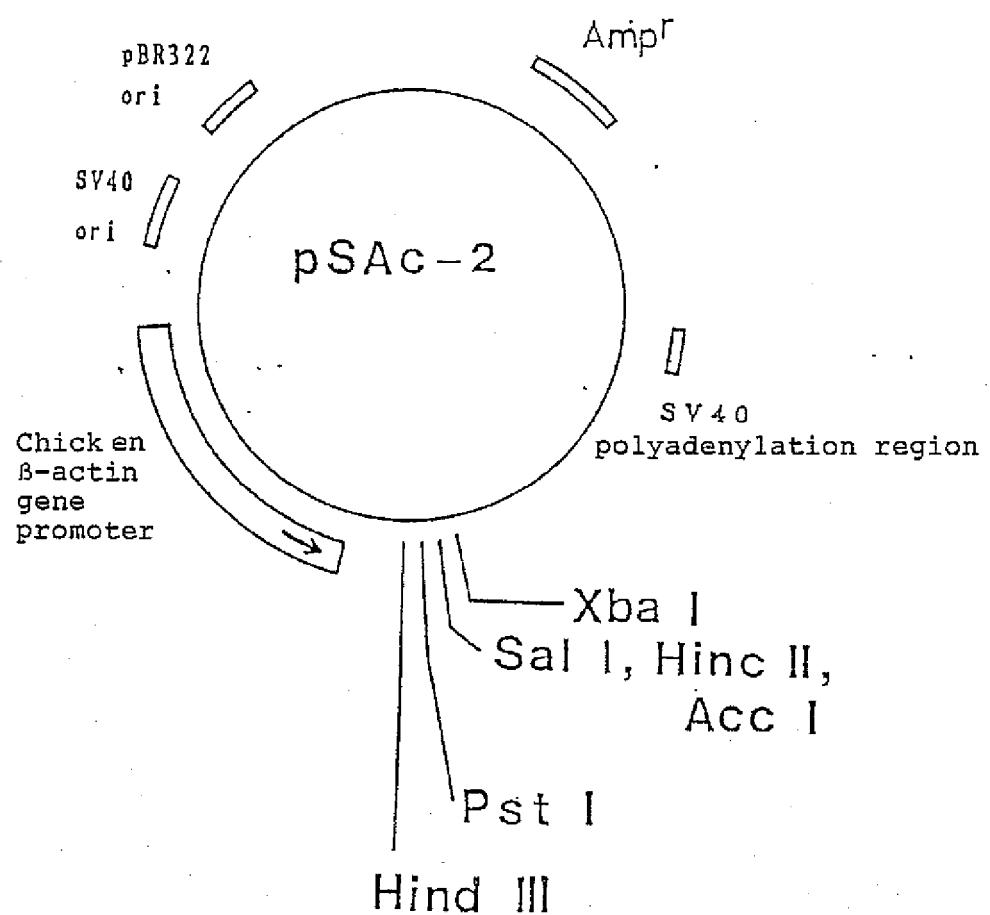


Fig. 8

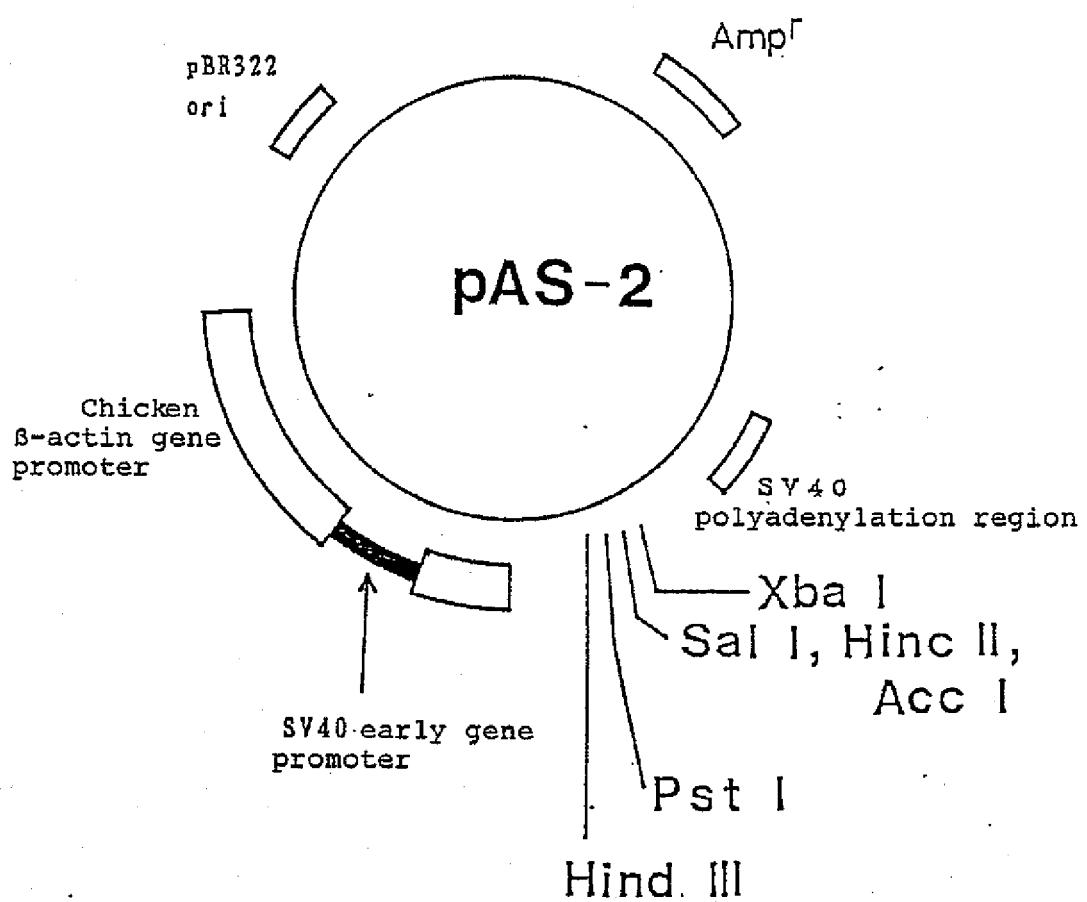


Fig. 9

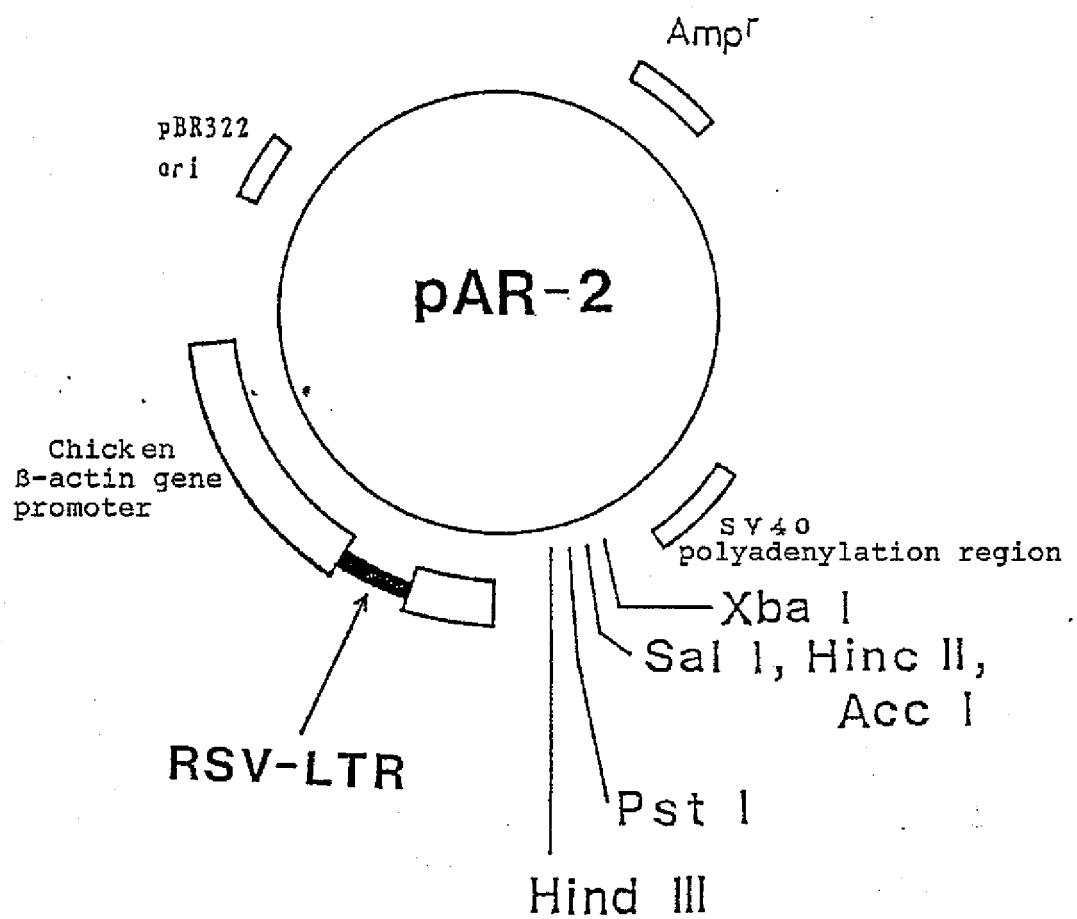


Fig. 10

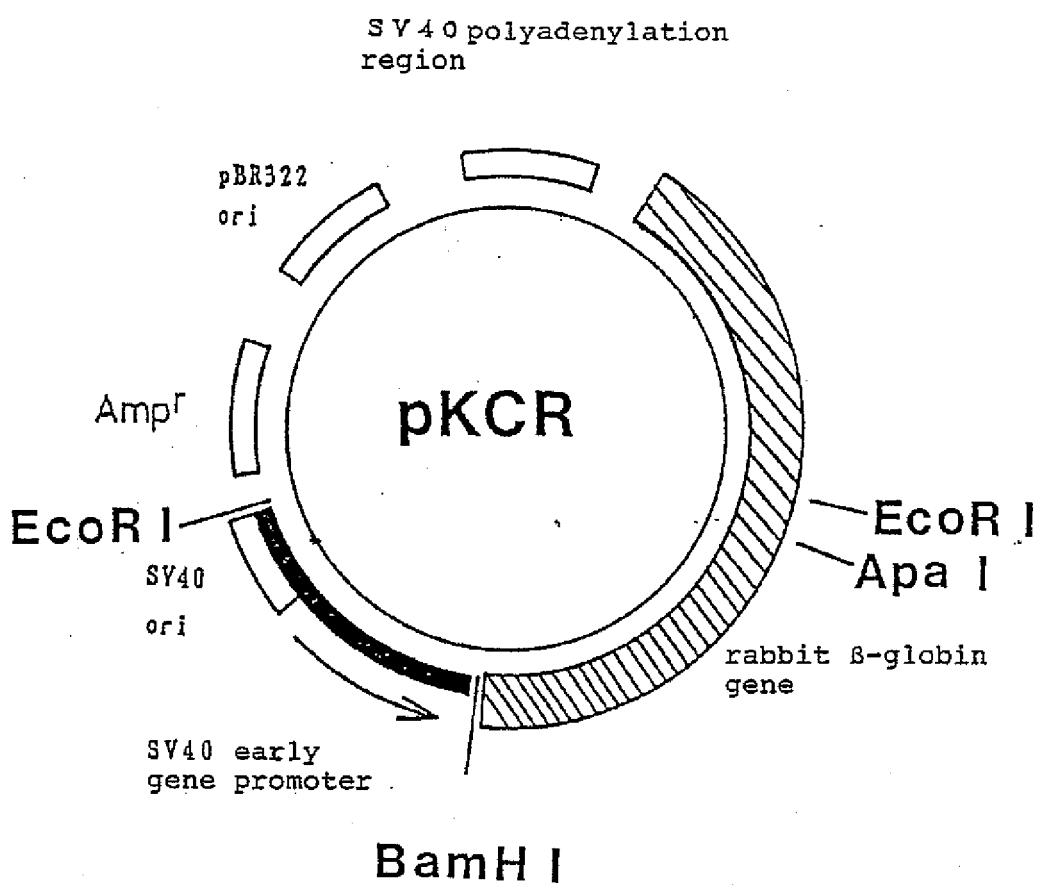


Fig. 11

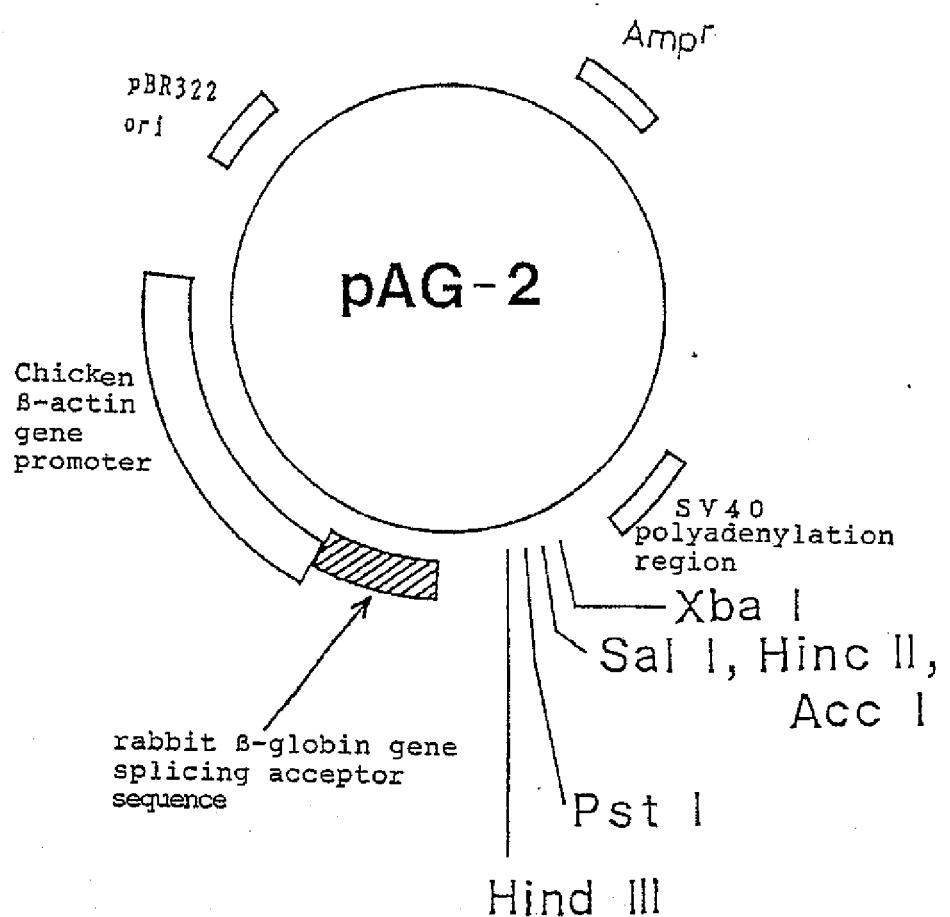


Fig. 12

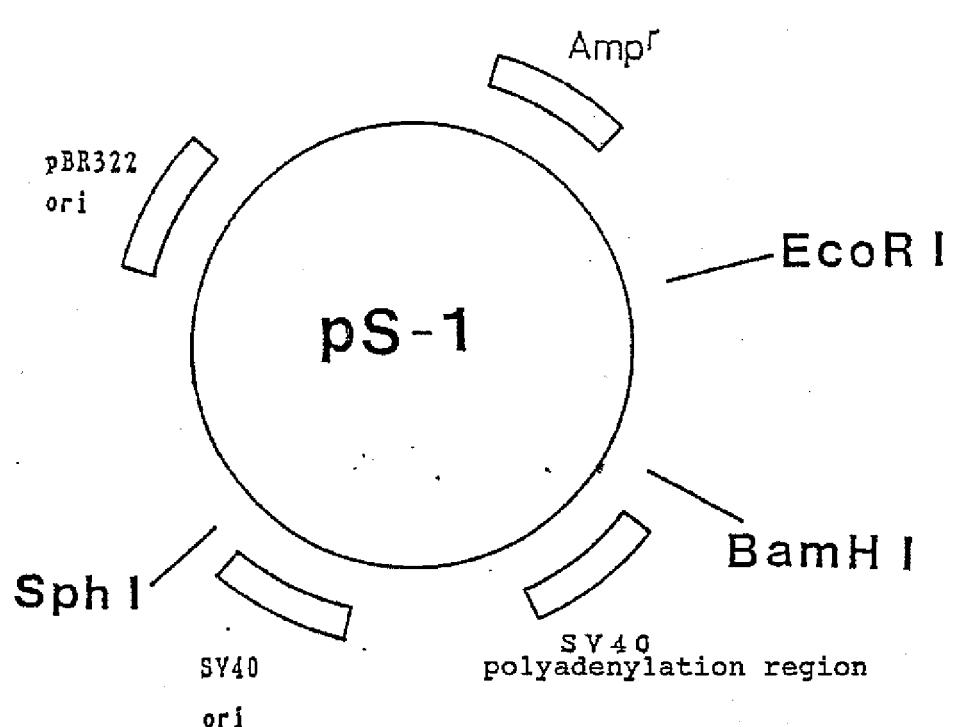


Fig. 13

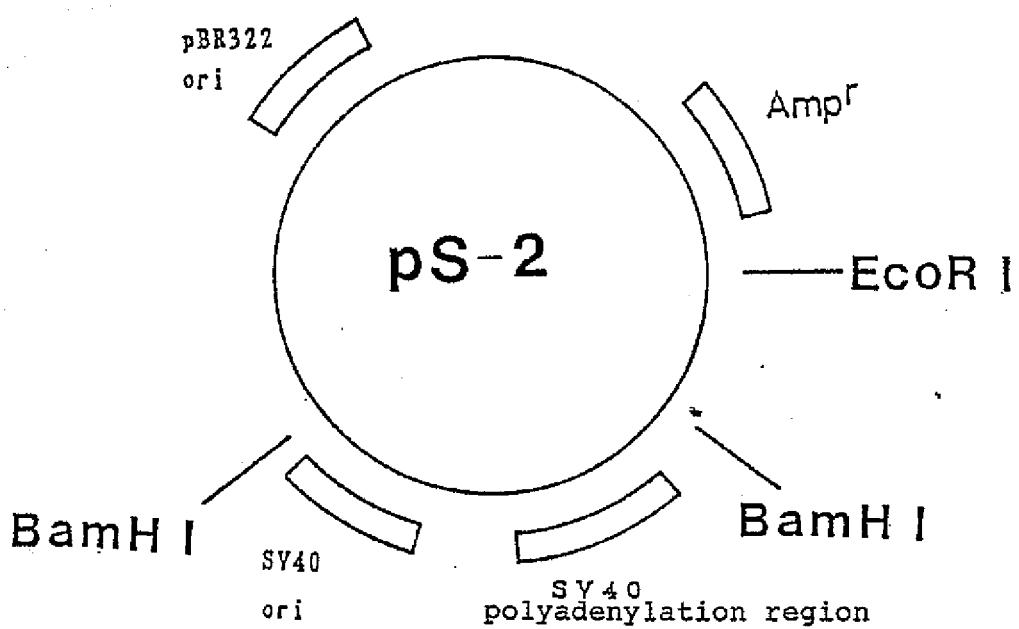


Fig. 14

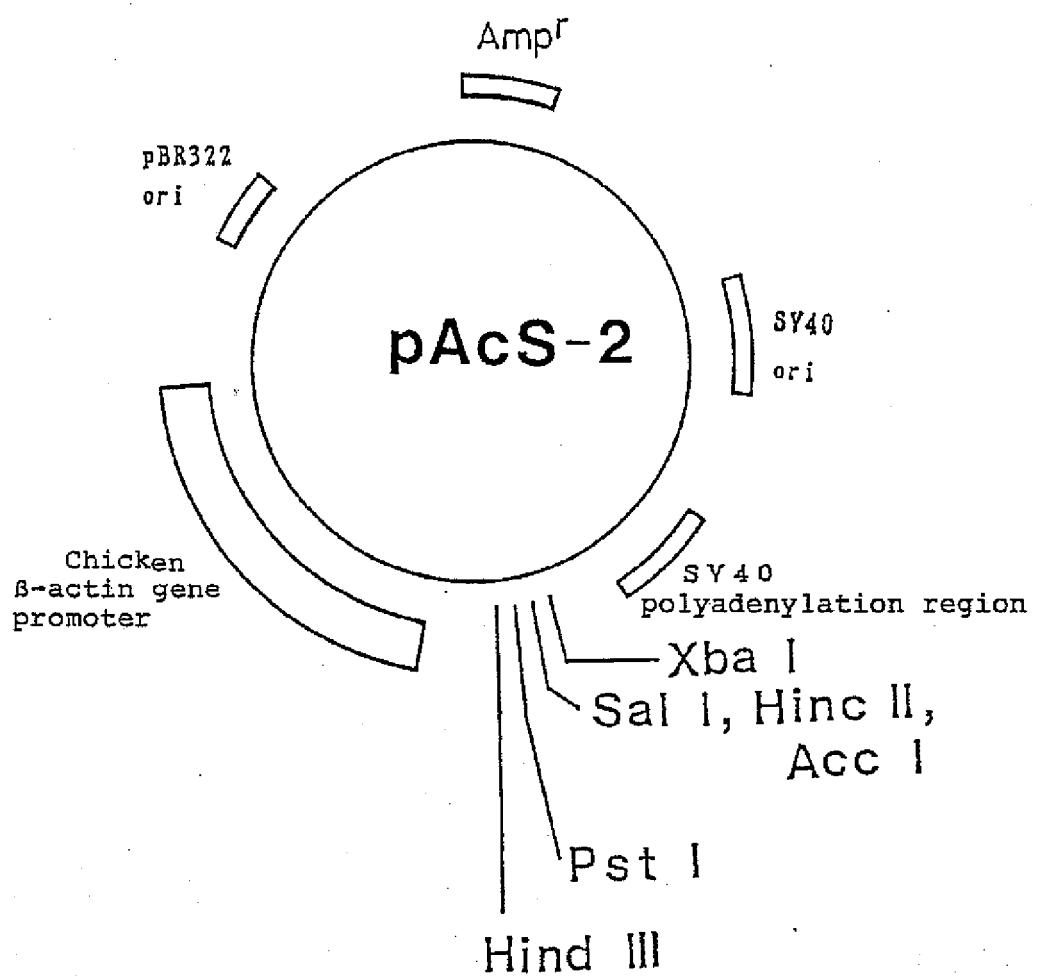


Fig. 15

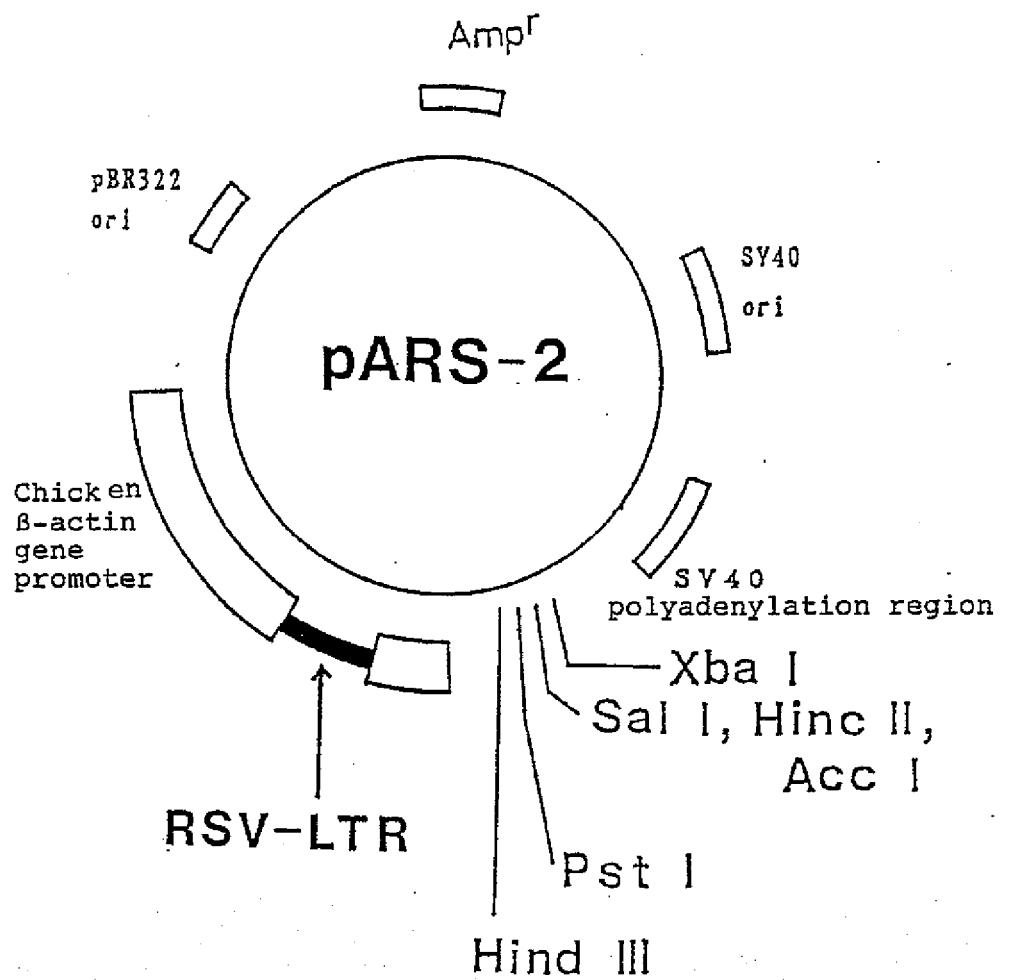


Fig. 16

